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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

003300-589

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

Unassigned

09/403269

INTERNATIONAL APPLICATION NO.  
PCT/SE98/00703INTERNATIONAL FILING DATE  
17 April 1998PRIORITY DATE CLAIMED  
18 April 1997

## TITLE OF INVENTION

DNA SEQUENCE CODING FOR A MAMMALIAN GLUCURONYL C5-EPIMERASE AND A PROCESS FOR ITS PRODUCTION

## APPLICANT(S) FOR DO/EO/US

Ulf Lindahl and Jin-ping Li

**It is contemplated that this Application be prosecuted using Claims 1 to 8 as submitted on May 25, 1999 during the international phase of prosecution and as further amended in the Preliminary Amendment filed herewith.**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).


Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

International Preliminary Examination Report

Response to Written Opinion filed May 25, 1999 with amended claims

A certified copy of the priority applications, Sweden Patent Application No. 9701454-2, filed 18 April 1997, was duly filed in connection with PCT/SE98/00703 and was received by DO/EO/US. Thus, it is believed that the priority claim has been properly substantiated.

U.S. APPLICATION NO. <b>09/403269</b> Unassigned		INTERNATIONAL APPLICATION NO. PCT/SE98/00703		ATTORNEY'S DOCKET NUMBER 003300-589	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00 (970) International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... \$670.00 (956) No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$760.00 (958) Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$970.00 (960) International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$96.00 (962)					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$ 970.00	
Surcharge of \$130.00 (154) for furnishing the oath or declaration later than 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 0.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	20 -20 =	0	X\$18.00 (966)	\$ 0.00	
Independent Claims	1 -3 =	0	X\$78.00 (964)	\$ 0.00	
Multiple dependent claim(s) (if applicable)			+ \$260.00(968)	\$ 0.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 970.00	
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$ 0.00	
<b>SUBTOTAL =</b>				\$ 970.00	
Processing fee of \$130.00 (156) for furnishing the English translation later than 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$ 0.00	
<b>TOTAL NATIONAL FEE =</b>				\$ 970.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). per property +				\$ 0.00	
<b>TOTAL FEES ENCLOSED =</b>				\$ 970.00	
				Amount to be: refunded	\$
				charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>970.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>02-4800</u> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>02-4800</u> . A duplicate copy of this sheet is enclosed.					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO:					
Benton S. Duffett, Jr. BURNS, DOANE, SWECKER & MATHIS, L.L.P. P.O. Box 1404 Alexandria, Virginia 22313-1404					
				 SIGNATURE	
				Benton S. Duffett, Jr. NAME	
				<u>22,030</u> REGISTRATION NUMBER	

09/403269  
514 Rec'd PCT/PTO 18 OCT 1999

PATENT  
Attorney Docket No. 003300-589

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of	)	<b>BOX PCT</b>
	)	
Ulf Lindahl and Jin-ping Li	)	Attn: DO/EO/US
	)	
Serial No. (corresponds to PCT/SE98/00703)	)	
	)	
Filed: October 18, 1999	)	Group Art Unit: Unassigned
	)	
For: DNA SEQUENCE CODING FOR A	)	Examiner: Unassigned
MAMMALIAN GLUCURONYL	)	
C5-EPIMERASE AND A PROCESS	)	
FOR ITS PRODUCTION	)	

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231  
Sir:

This application corresponds to PCT/SE98/00703.

It is contemplated that this Application be prosecuted using Claims 1 to 8 as submitted on May 25, 1999 during the international phase of prosecution and as further herein.

Please amend the above-identified Application as indicated.

**In the Abstract of the Disclosure**

Please add the Abstract of the Disclosure that is provided herewith on a separate sheet.

**In the Claims**

Claim 4, line 3, delete "any one of the preceding claims" and insert --claim 1--.

Claim 6, line 2, delete "or 5".

Claim 7, line 6, delete "or 5".

Please add the following new Claims 9 to 20:

--9. A recombinant expression vector containing a transcription unit comprising a DNA sequence according to claim 2, a transcriptional promoter, and a polyadenylation sequence.

10. A recombinant expression vector containing a transcription unit comprising a DNA sequence according to claim 3, a transcriptional promoter, and a polyadenylation sequence.

11. A recombinant expression vector according to claim 9, characterized in that the vector is a Baculovirus.

12. A recombinant expression vector according to claim 10, characterized in that the vector is a Baculovirus.

13. A host cell transformed with the recombinant expression vector of claim 5.

14. A host cell transformed with the recombinant expression vector of claim 9.

15. A host cell transformed with the recombinant expression vector of claim 10.

16. A process for the manufacture of a glucuronyl C5-epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid

(IdoA), comprising cultivation of a host cell transformed with a recombinant expression vector according to claim 5 in a nutrient medium allowing expression and secretion of said epimerase or functional derivative thereof.

17. A process for the manufacture of a glucuronyl C5-epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), comprising cultivation of a host cell transformed with a recombinant expression vector according to claim 9 in a nutrient medium allowing expression and secretion of said epimerase or functional derivative thereof.

18. A process for the manufacture of a glucuronyl C5-epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), comprising cultivation of a host cell transformed with a recombinant expression vector according to claim 10 in a nutrient medium allowing expression and secretion of said epimerase or functional derivative thereof.

19. A glucuronyl C5-epimerase or a functional derivative thereof whenever prepared by the process of claim 16.

20. A glucuronyl C5-epimerase or a functional derivative thereof whenever prepared by the process of claim 17.--

REMARKS

The present amendment adds an Abstract of the Disclosure and removes the multiple dependency from the claims.

The examination and allowance of the application are respectfully requested.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Benton S. Duffett Jr.  
Benton S. Duffett, Jr.  
Registration No. 22,030

P.O. Box 1404  
Alexandria, Virginia 22313-1404  
(703) 836-6620

Filed: October 18, 1999

[illegible]

## DNA SEQUENCE CODING FOR A MAMMALIAN GLUCURONYL C5-EPIMERASE AND A PROCESS FOR ITS PRODUCTION

The present invention relates to an isolated or recombinant DNA sequence coding for a glucuronyl C5-epimerase capable of converting D-glucuronic acid to L-iduronic acid. The invention also relates to a process  
5 for the manufacture of such epimerase.

Background of the invention

Heparin and heparan sulfate are complex, sulfated glycosaminoglycans composed of alternating glucosamine  
10 and hexuronic acid residues. The two polysaccharides are structurally related but differ in composition, such that heparin is more heavily sulfated and shows a higher ratio of L-iduronic acid (IdoA)/D-glucuronic acid (GlcA) units (Kjellén, L. and Lindahl, U. (1991) Annual Review of Bio-  
15 chemistry 60, 443-475; Salmivirta, M., Lidholt, K. and Lindahl, U. (1996) The FASEB Journal 10, 1270-1279). Heparin is mainly produced by connective tissue-type mast cells, whereas heparan sulfate has a ubiquitous distribu-  
20 tion and appears to be expressed by most cell types. The biological roles of heparin and heparan sulfate are presumably largely due to interactions of the polysaccharides with proteins, such as enzymes, enzyme inhibitors, extracellular-matrix proteins, growth factors/cytokines and others (Salmivirta, M., Lidholt, K. and Lindahl, U.  
25 (1996) The FASEB Journal 10, 1270-1279). The interactions tend to be more or less selective/specific with regard to carbohydrate structure, and thus depend on the amounts and distribution of the various sulfate groups and hexuronic acid units. Notably, IdoA units are believed to ge-  
30 nerally promote binding of heparin and heparan sulfate chains to proteins, due to the marked conformational flexibility of these residues (Casu, E., Petitou, M., Provasoli, M. and Sinay, P. (1988) Trends in Biochemical Sciences 13, 221-225).

Heparin and heparan sulfate are synthesized as proteoglycans. The process is initiated by glycosylation reactions that generate saccharide sequences composed of alternating GlcA and N-acetylglucosamine (GlcNAc) units covalently bound to peptide core structures. The resulting (GlcA $\beta$ 1,4-GlcNAc $\alpha$ 1,4-)<sub>n</sub> disaccharide repeats are modified, probably along with chain elongation, by a series of enzymatic reactions that is initiated by N-deacetylation and N-sulfation of GlcNAc units, continues through C-5 epimerization of GlcA to IdoA residues, and is concluded by the incorporation of O-sulfate groups at various positions. The N-deacetylation/N-sulfation step has a key role in determining the overall extent of modification of the polymer chain, since the GlcA C-5 epimerase as well as the various O-sulfotransferases all depend on the presence of N-sulfate groups for substrate recognition. While the GlcNAc N-deacetylation and N-sulfation reactions are both catalyzed by the same protein, isolation and molecular cloning of N-deacetylase/N-sulfotransferase from different tissue sources implicated two distinct forms of the enzyme. The two enzyme types differ with regard to kinetic properties, and it has been suggested that they may be differentially involved in the biosynthesis of heparin and heparan sulfate.

#### Summary of the invention

The present invention provides for an isolated or recombinant DNA-sequence coding for a mammalian, including human, glucuronyl C-5 epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA).

The invention also provides for a recombinant expression vector containing a transcription unit comprising a DNA sequence as described above, a transcriptional promoter, and a polyadenylation sequence.

The invention also provides for a process for the manufacture of a glucuronyl C-5 epimerase or a functional

derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), comprising cultivation of a cell line transformed with the above recombinant expression vector in a nutrient medium allowing expression and secretion of said epimerase or functional derivative thereof.

Specific DNA sequences according to the invention are defined in appended claims 2, 3 and 4.

Furthermore, the invention provides for a host cell transformed with such recombinant expression vector.

Finally, the invention covers a glucuronyl C-5 epimerase or a functional derivative thereof whenever prepared by the process outlined above.

Brief description of the appended figures and sequence listing

**Sequence listing: Nucleotide sequence and the predicted amino acid sequence of the C5-epimerase.** The predicted amino acid sequence is shown below the nucleotide sequence. The numbers on the right indicate the nucleotide residue and the amino acid residue in the respective sequence. The five sequenced peptides appear in **bold**. The N-terminal sequence of the purified protein is shown in **bold and italics**. The potential N-glycosylation sites (\*) are shown. The potential transmembrane region is underlined.

**Fig 1. In vitro transcription-translation.** The epimerase cDNA was inserted into a pcDNA3 expression vector and linearized with XbaI at the 3'-end. It was then subjected to in vitro transcription-translation in a rabbit reticulocyte lysate system in the presence of [<sup>35</sup>S]methionine, as described in "Experimental Procedures". The translation product of epimerase cDNA (Epi) has a molecular weight of ~50 kDa, by comparison with the LMW protein standard. A 118 kDa control sample of  $\beta$ -galactosidase

(C), expressed in the same system, is shown for comparison.

**Fig 2. Effect of the expressed C5-epimerase on N-**

5 **deacetylated, N-sulfated capsular polysaccharide from E.**  
**coli K5.** Metabolically  $^3\text{H}$ -labeled K5 polysaccharide was  
N-deacetylated and N-sulfated, and was then incubated  
with (A) lysate of Sf9 cells infected with recombinant  
C5-epimerase; (B) lysate of Sf9 cells infected with re-  
10 combinant  $\beta$ -glucuronidase. The incubation products were  
treated with  $\text{HNO}_2 / \text{NaBH}_4$ , and the resultant hexuronyl-  
anhydromannitol disaccharides were recovered and sepa-  
rated by paper chromatography. The arrowheads indicate  
the migration positions of glucuronosyl-anhydromannitol  
15 (GM) and iduronosyl-anhydromannitol (IM) disaccharide  
standards. For further information see "Experimental Pro-  
cedures".

**Fig 3. Northern analysis of C5-epimerase mRNA expressed**

20 **in bovine lung and mastocytoma cells.** Total RNA from  
each tissue/cell line was separated by agarose gel elec-  
trophoresis. A blot was prepared, probed with a  $^{32}\text{P}$ -  
labeled 2460-bp fragment of the epimerase cDNA clone, and  
finally exposed to X-ray film. (Kodak, Amersham). The ar-  
25 row indicates the positions of molecular standards. For  
further information see "Experimental Procedures".

Detailed description of the invention

The present invention relates to DNA sequences cod-  
30 ing for a mammalian glucuronyl C5-epimerase or a func-  
tional derivative thereof, such epimerase or derivative  
being capable of converting D-glucuronic acid (GlcA) to  
L-iduronic acid (IdoA). The term "mammalian" is intended  
to include also human varieties of the enzyme.

35 As used herein the definition "glucuronyl C5-  
epimerase or a functional derivative thereof" refers to  
enzymes which have the capability of converting D-

glucuronic acid to L-iduronic acid. Accordingly, the definition embraces all epimerases having such capability including functional variants, such as functional fragments, mutants resulting from mutageneses or other recombinant techniques. Furthermore, the definition is intended to include glycosylated or unglycosylated mammalian glucuronyl C5-epimerases, polymorphic or allelic variants and other isoforms of the enzyme. "Functional derivatives" of the enzyme can include functional fragments, functional fusion proteins or functional mutant proteins. Such epimerases included in the present invention can have a deletion of one or more amino acids, such deletion being an N-terminal, C-terminal or internal deletion. Also truncated forms are envisioned as long as they have the conversion capability indicated herein.

Operable fragments, mutants or truncated forms can suitably be identified by screening. This is made possible by deletion of for example N-terminal, C-terminal or internal regions of the protein in a step-wise fashion, and the resulting derivative can be analyzed with regard to its capability of the desired conversion of D-glucuronic acid to L-iduronic acid. If the derivative in question operates in this capacity it is considered to constitute a functional derivative of the epimerase proper.

Examples of useful epimerases are proteins having the sequence as shown in the sequence listing or substantially as shown in the sequence listing and functional portions thereof.

#### EXPERIMENTAL PROCEDURES

*Peptide Purification and Sequencing* - The 52 kDa epimerase protein (~1µg), purified from a detergent extract of bovine liver by chromatography on O-desulfated heparin-Sepharose, Red-Sepharose, Phenyl-Sepharose, and Concanavalin A-Sepharose (Campbell, P., Hannesson, H.H., Sandbäck, D., Rodén, L., Lindahl, U. and Li, J.-p. (1994)

J Biol Chem 269, 26953-26958), was subjected to direct N-terminal sequencing using a model 470A protein sequenator (Applied Biosystems) equipped with an on-line 120 phenylthiohydantoin analyzer (Tempst, P., and Riviere, L. (1989) Anal. Biochem. 183, 290-300). Another sample (~1µg) was applied to preparative (12%) SDS-PAGE and was then transferred to a PVDF membrane. After staining the membrane with Coomassie Blue, the enzyme band was excised. Half of the material was submitted to direct N-terminal sequence analysis, whereas the remainder was digested with Lys-C (0.0075 U; Waco) in the presence of 1% RTX-100/10% acetonitrile/100mM Tris-HCl, pH 8.0. The generated peptides were separated on a reverse phase C4-column, eluted at a flow rate of 100 µl/min with a 6-ml 10-70% acetonitrile gradient in 0.1% trifluoroacetic acid, and detected with a 990 Waters diode-array detector. Selected peptides were then subjected to sequence analysis as described above.

*Probes for Screening* - Total RNA was extracted from bovine liver according to the procedures of Sambrook et al. (1989). Single-stranded cDNA was synthesized by incubating ~5 µg of bovine liver total RNA (denatured at 65°C, 3 min) with a reaction mixture containing 1 unit RNase inhibitor (Perkin-Elmer Corp.), 1 mM of each dNTP, 5 µM random nucleotide hexamer and 1.25 units of murine leukemia virus reverse transcriptase (Perkin-Elmer Corp.) in a buffer of 10 mM Tris-HCl, pH 8.3. The mixture was kept at 42°C for 45 min and then at 95°C for 5 min. Degenerated oligonucleotide primers were designed based on the amino-acid sequence determined for one of the internal peptides derived from the purified epimerase (Table I). Single-stranded bovine liver cDNA was applied to PCR together with 100 pmols of primers 1 (sense) and 3 (antisense), in a total volume of 100 µl containing 1µl of 10% Tween 20, 6 mM MgCl<sub>2</sub>, 1 mM of each dNTP, and 2.5 units Taq polymerase (Pharmacia Biotech) in a buffer of 10 mM Tris-HCl, pH 9.0. The reaction products were sepa-

rated on a 12% polyacrylamide gel. A ~100-bp band was cut out from the gel and reamplified using the same PCR conditions. After an additional polyacrylamide gel electrophoresis, the product was isolated and sequenced, yielding a 108-bp sequence. This PCR product was subcloned into a pUC119 plasmid. The DNA fragment cleaved from the plasmid was labeled with [<sup>32</sup>P]dCTP (DuPont NEN) using a Randon Primed DNA Labeling Kit (Boehringer Mannheim).

10        *Screening of cDNA Library* - A bovine lung cDNA library constructed in a lgt10 vector (Clontech) was screened with the 108-bp PCR fragment as hybridizing probe. The nitrocellulose replicas of the library plaques were prehybridized in 6xSSC, 5xDenhart's solution containing 0.1% SDS and 0.1 mg/ml denatured salmon DNA for 2 hours at 65°C. Hybridization was carried out at 42°C in the same solution containing <sup>32</sup>P-labeled probe for 16-18 hours. The filters were washed two times with 2x SSC, 0.5% SDS and two times with 0.5x SSC, 1% SDS at the same temperature. The library was repeatedly screened twice under the same conditions. Finally, the entire cDNA phage library was subjected to PCR amplification using lgt10 forward and reverse primers (Clontech) with a epimerase cDNA specific primer (5'-GCTGATTCTTTTCTGTC-3').

25        *Subcloning and Sequencing of cDNA Inserts* - cDNA inserts, isolated by preparative agarose gel electrophoresis (Sambrook et al., 1989) after EcoRI restriction cleavage of recombinant bacteriophage DNA, were subcloned into a pUC119 plasmid. The complete nucleotide sequence was determined independently on both strands using the dideoxy chain termination reaction either with [<sup>35</sup>S]dATP and the modified T7 DNA polymerase (Sequenase version 2.0 DNA Sequencing Kit; U. S. Biochemical Corp.) or the ALFT<sup>TM</sup> System (Pharmacia Biotech). DNA sequences were compiled and analyzed using the DNASTAR<sup>TM</sup> program (Lasergene).

35        *Polyclonal Antibodies and Immunodetection* - A peptide corresponding to residues 77 - 97 of the deduced epime-

rase amino-acid sequence was chemically synthesized (Åke Engström, Department of Medical and Physiological Chemistry, Uppsala University, Sweden), and was then conjugated to ovalbumin using glutaraldehyde (Harlow, E. and Lane, D. (1989) in Antibodies: A Laboratory Manual, pp 78-79, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). A rabbit was immunized with the peptide conjugates together with Freund's adjuvant. After 6 boosts (each with 240 µg conjugated peptide) blood was collected and the serum recovered. The antibody fraction was further purified on a Protein A-Sepharose column (Pharmacia Biotech), and used for immunoblotting.

Samples of GlcA C5-epimerase were separated under denaturing conditions by 12% SDS-PAGE, and were then transferred to a nitrocellulose membrane (Hybond™ ECL). ECL immunoblotting was performed according to the protocol of the manufacturer (Amersham). Briefly, the membrane was first treated with blocking agent, then incubated with purified antibody, and finally incubated with the peroxidase labeled anti-rabbit antibody. After adding the ECL reagent, the light emitted by the chemical reaction was detected by exposure to Hyperfilm™ ECL for 30-60 sec.

*Northern Blot Hybridization* -- Bovine liver and lung total RNA was prepared according to Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY), and mouse matocytoma (MCT) total RNA was extracted from a tumor cell line (Montgomery, R.I., Lidholt, K., Flay, N.W., Liang, J., Verter, B., Lindahl, U. and Esko, J.D. (1992) PNAS **89**, 11327-113331) as described by Chomczynski and Sacci (1987). Total RNA from each tissue (~20 µg samples) was denatured in 50% formamide (v/v), 5% formaldehyde, 20 mM Mops buffer, pH 7.0, at 65 °C for 5 min. The denatured RNA was separated by electrophoresis in 1.2% agarose gel containing 5% formaldehyde (v/v), and was then transferred to a Hybond N<sup>+</sup> nylon membrane (Amersham). The RNA blot was pre-hybr-

dized in ExpressHyb Hybridization Solution (Clontech) at 65 °C for 1 h, and subsequently hybridized in the same solution with-a [<sup>32</sup>P]dCTP-labeled DNA probe (a 2460 bp fragment including the 5'-end of the cDNA clone; see the sequence listing). The membrane was washed in 2x SSC, 0.5% SDS at the same temperature for 2 x 15 min and in 0.5x SSC, 0.5 % SDS for 2 x 15 min. The membrane was exposed to a Kodak X-ray film at -70°C for 24h.

*In Vitro Translation* - The 3-kb GlcA C5-epimerase clone, inserted in a pcDNA3 expression vector (Invitrogen) was linearized at the 3'-end by restriction enzyme XbaI. In vitro translation was carried out with a Linked T7 transcription-translation system (Amersham) according to the instructions of the manufacturer. The corresponding mRNA generated by incubation of 0.5 µg linearized plasmid DNA with a T7 polymerase transcription mix (total volume, 10 µl; 30°C; 15 min) was mixed with an optimized rabbit reticulocyte lysate containing 50µCi [<sup>35</sup>S]methionine (total volume, 50 µl), and further incubated at 30 °C for 1 h. A sample (5 µl) of the product was subjected to 12% SDS-PAGE. The gel was directly exposed to a Kodak X-ray film. After exposure, the applied protein molecular standards ( LMW Molecular Calibration Kit, Pharmacia Biotech) were visualized by staining the gel with Coomassie Blue.

*Expression of the GlcA C5-Epimerase* - The GlcA C5-epimerase was expressed using a BacPAK8™ Baculovirus Expression System (Clontech), according to the instructions by the manufacturer. Two oligonucleotides, one at the 5'-end of the cDNA clone (1-17 bp, sense) and the other at the 3'-end of the coding sequence (1387-1404 bp, antisense), were used to PCR amplify the coding sequence of the C5-epimerase cDNA clone. The resulting fragment was cloned into the BacPAK8 vector. Sf9 insect cells, maintained in Grece's Insect Medium (GibcoBRL) supplemented with 10% fetal calf serum and penicillin/streptomycin, were then cotransfected by the C5-epimerase construct

along with viral DNA. Control transfections were performed with constructs of a  $\beta$ -glucuronidase cDNA construct included in the expression kit, and a mouse cDNA coding for the GlcNAc N-deacetylase/N-sulfotransferase implicated in heparin biosynthesis (Eriksson, I., Sandbäck, D., Ek, B., Lindahl, U. and Kjellén, K. (1994) J.Biol. Chem. **269**, 10438-10443; Cheung, W.F., Eriksson, I., Kusche-Gullberg, M., Lindahl, U. and Kjellén, L. (1996) Biochemistry **35**, 5250-5256). Single plaques of each co-transfected recombinant were picked and propagated. Two Petri dishes (60-mm) of Sf9 cells were infected by each recombinant virus stock and incubated at 27°C for 5 days. The cells from one dish were used for total RNA extraction and Northern analysis performed as described above. Cells from the other dish were lysed in a buffer of 100 mM KCl, 15 mM EDTA, 1% Triton X-100, 50 mM HEPES, pH 7.4, containing 1mM PMSF and 10 $\mu$ g/ml pepstatin A. Supernatants of cell lysates as well as conditioned media were analyzed for epimerase activity. Protein contents of the cell lysates were estimated by the method of Bradford (1976) or by the BCA reagent procedure (Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. biochem **150**, 76-85).

*Demonstration of GlcA C5-epimerase activity* - Epimerase activity was assayed using a biphasic liquid scintillation counting procedure, essentially as described by Campbell et al. (1994) above. The reaction mixtures, total volume 55  $\mu$ l, contained 25  $\mu$ l cell lysate or medium, 25  $\mu$ l of 2x epimerase assay buffer (20 mM HEPES, 30 mM EDTA, 0.02% Triton X-100, 200 mM KCl, pH 7.4) and 5  $\mu$ l of substrate (10,000 cpm  $^3$ H). The substrate was a chemically N-deacetylated and N-sulfated polysaccharide, obtained from *E. coli* K5 according to the procedure of Campbell et al. (1994), except that D-[5- $^3$ H]glucose was substituted for D-[1- $^3$ H]glucose.

Enzymatic conversion of D-glucuronic to L-iduronic acid was demonstrated using the metabolically  $1\text{-}^3\text{H}$ -labeled substrate (N-deacetylated, N-sulfated capsular polysaccharide from *E. coli* K5) and the analytical procedure described by Campbell et al. (1994). A sample ( $\sim 20\text{ }\mu\text{g}$ ; 200,000 cpm of  $^3\text{H}$ ) of the modified polymer was incubated with 250  $\mu\text{l}$  of cell lysate in a total volume of 300  $\mu\text{l}$  epimerase assay buffer at  $37^\circ\text{C}$  for 6 hours. The incubation was terminated by heating at  $100^\circ\text{C}$  for 5 min. The sample was mixed with 50 $\mu\text{g}$  of carrier heparin and reacted with nitrous acid at pH 1.5 (Shively, J., and Conrad, H.E. (1976) Biochemistry 15, 3932-3942), followed by reduction of the products with  $\text{NaBH}_4$ . The resultant hexuronyl-anhydromannitol disaccharides were recovered by gel chromatography on a column (1 x 200 cm) of Sephadex G-15 in 0.2 M  $\text{NH}_4\text{HCO}_3$ , lyophilized, and subjected to paper chromatography on Whatman No 3MM paper in ethyl acetate/acetic acid/water (3:1:1).

## 20 RESULTS

*Generation of a Probe and Screening of cDNA Library*  
- Amino acid sequence data for the  $\sim 52\text{ kDa}$  protein were obtained by digesting highly purified epimerase with lysine-specific protease, followed by separation of the generated peptides on a reverse phase column. The five most prominent peptides were isolated and subjected to amino-acid sequencing (Table I). One of the peptides (peptide 1) was found to correspond to the N-terminal sequence of the native protein. The sequence of the largest peptide obtained (peptide 5 in Table I), was used to design two sense and one antisense degenerate oligonucleotide primers, as shown in Table I. A DNA probe was produced by PCR using primers 1 and 3 with bovine liver cDNA as template. The resultant  $\sim 100\text{ bp}$  DNA fragment was purified by polyacrylamide gel electrophoresis, reamplified using the same primers, and finally isolated by electrophoresis. The identity of the product was ascertained by

"nested" PCR, using primers 2 and 3, which yielded the expected ~60 bp fragment (data not shown). Moreover, sequencing of the larger (108 bp) DNA fragment gave a deduced amino-acid sequence identical to that of the isolated peptide (Table I).

The 108-bp PCR product was labeled with [<sup>32</sup>P]dCTP and used for screening of a bovine lung lgt10 library. One hybridizing clone, containing a 3-kb insert, was identified. Repeated screening of the same library yielded two additional positive clones, both of which were of smaller size. Subsequent sequencing showed both of the latter clones to be contained within the 3.0-kb species (data not shown). The 3-kb clone was sequenced through both strands and was found to contain altogether 3073 bp; an additional 12-bp sequence was added at the 5'-end through characterization of a separate clone obtained by PCR amplification of the phage library (see "Experimental Procedures").

#### *Characterization of cDNA and Predicted Protein*

**Structure** - The total cDNA sequence identified, in all 3085 bp, contains an open reading frame corresponding to 444 amino-acid residues (the sequence listing). Notably, the coding region (1332 bp) is heavily shifted toward the 5'-end of the available cDNA, and is flanked toward the 3'-end by a larger (1681 bp) noncoding segment. The deduced amino-acid sequence corresponds to a 49,905 dalton polypeptide. All of the five peptides isolated after endo-peptidase digestion (Table I) were recognized in the primary structure deduced from the cDNA (the sequence listing). One of these peptides (peptide 1) is identical to the N-terminus of the isolated liver protein. This peptide was found to match residues 74 - 86 of the deduced polypeptide sequence. The enzyme isolated from bovine liver thus represents a truncated form of the native protein.

Generation of mRNA from an expression vector inserted with the 3-kb cDNA clone, followed by incubation

of the product with rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine, resulted in the formation of a distinct labeled protein with an estimated M<sub>r</sub> of ~50kDa (Fig. 1). This product was recognized in immunoblotting (data not shown) by polyclonal antibodies raised against a synthetic peptide corresponding to residues 77 - 97 (see the sequence listing) of the deduced amino-acid sequence. The same antibodies also reacted with the isolated ~52 kDa bovine liver protein (data not shown). These observations establish that the 3-kb cDNA is derived from the transcript that encodes the isolated ~52 kDa bovine liver protein.

The cDNA structure indicates the occurrence of 3 potential N-glycosylation sites (the sequence listing). Sugar substituents may be important for the proper folding and catalytic activity of the enzyme, since the protein expressed in bacteria (which also gave a strong Western signal towards the polyclonal antibodies raised against the synthetic peptide; data not shown) was devoid of enzymatic activity. A potential transmembrane region is underlined in the sequence listing. The predicted protein contains two cystein residues, only one of which occurs in the isolated (truncated) protein. Since NEM was inhibitory to epimerase activity (data not shown), this single cystein unit may be essential to the catalytic mechanism.

*Functional Expression of the GlcA C5-Epimerase* - A variety of expression systems were tested in attempts at generating the cloned protein in catalytically active form. A protein obtained by in vitro translation using a rabbit reticulocyte lysate system (see Fig. 1) showed no detectable epimerase activity. A construct made by inserting the 3-kb cDNA into a pCDNA3 vector (Invitrogen) failed to induce mRNA formation (or translation) in any of the cell lines tested (human embryonic kidney (293), COS-1 or CHO cells) (data not shown). We also attempted to express the enzyme in a bacterial pET system

(Novagen). The transformed bacteria yielded appreciable amounts of immunoreactive protein which, however, lacked detectable enzyme activity (data not shown).

Cotransfection of epimerase recombinant with baculovirus into Sf9 insect cells resulted in the generation of abundant GlcA C5-epimerase activity (Table II). In two separate experiments, the lysates from cells infected with the same epimerase recombinant virus stock showed >10-fold higher enzyme activities, on a mg protein basis, than the corresponding fractions from cells infected with control recombinant virus stock. The conditioned media of cells infected with epimerase recombinant showed 20- 30-fold higher enzyme activities than the corresponding fractions from cells infected with control plasmid virus stock. Transfections with cDNA encoding other enzymes, such as a  $\beta$ -glucuronidase, or the mouse mastocytoma GlcNAc N-deacetylase/N-sulfotransferase involved in heparin biosynthesis (Eriksson et al., 1994), did not significantly increase the epimerase activity beyond control levels. Notably, the higher  $^3\text{H}_2\text{O}$  release recorded for control samples as compared to heat-inactivated expressed enzyme (Table II) suggests that the insect cells constitutively produce endogenous C5-epimerase.

The polysaccharide substrate used for routine assays of epimerase activity was obtained by chemically N-deacetylating and N-sulfating the capsular polysaccharide [(GlcA $\beta$ 1,4-GlcNAc $\alpha$ 1,4) $_n$ ] of *E. coli* K5 that had been grown in the presence of [5- $^3\text{H}$ ]glucose. The data in Table II thus reflect the release of  $^3\text{H}_2\text{O}$  from 5- $^3\text{H}$ -labeled GlcA units in the modified polysaccharide, due to enzyme action (Jacobsson, I., Bäckström, G., Höök, M., Lindahl, U., Feingold, D.S., Malmström, M. and Rodén, L. (1979) J. Biol. Chem. 254, 2975-2982; Jacobsson, I., Lindahl, U., Jensen, J.W., Rodén, L., Prihar, H. and Feingold, D.S. (1984) Journal of Biological Chemistry 259, 1056-1064). More direct evidence for the actual conversion of GlcA to IdoA residues was obtained by incubating the expressed

enzyme with an analogous substrate, obtained following incubation of the bacteria with [1-<sup>3</sup>H]glucose. This substrate will retain the label through the epimerization reaction, and can therefore be used to demonstrate the formation of IdoA-containing disaccharide units. Following incubation with the recombinant epimerase, 21% of the hexuronic acid residues was converted to IdoA, as demonstrated by paper chromatography of disaccharide deamination products (Fig. 2). The composition of the incubated polysaccharide thus approached the equilibrium ratio of IdoA/GlcA, previously determined to ~3/71).

Northern Analysis -Total RNA, from bovine liver, lung, and mouse mastocytoma, were analysed by hybridization with a 2460-bp DNA fragment from epimerase cDNA clone as a probe. Both bovine liver and lung gave identical transcription patterns, with a dominant transcript of ~9 kb and a weak ~5 kb band (Fig. 3). By contrast, the mastocytoma RNA showed only the ~5 kb transcript.

It is to be noted that the present invention is not restricted to the specific embodiments of the invention as described herein. The skilled artisan will easily recognize equivalent embodiments and such equivalents are intended to be encompassed in the scope of the appended claims.

Table I  
Peptide and primer sequences

A. N-terminal sequences of isolated C5-epimerase		
1. PNDWXVPKGCFFMA (free solution)		
2. PXDWTVPKGXF (band excised from PVDF-membrane)		
B. Peptide sequences		
1. PNDXTVPK		
2. XXIAPETSEGXSLQL		
3. GGWPIMVTRK		
4. FLSEQHGV		
5. <u>KAMLPLYDTGSGTIYDLRHFMLGIAPNLAXWDYHTT</u>		
primer 1 (sense)	primer 2 (sense)	primer 3 (antisense)
C. Primer <sup>a</sup>		
1 (S)	5'-cc gaattcAARGCNATGYTNCCNYT-3' <sup>b</sup>	Degeneracy 384
2 (S)	5'-cc gaattcGAYYTNMGNCAYTTYATG-3'	288
3 (AS)	5'-cc ggatccGTNGTRTGRTARTCCCA-3'	32

<sup>a</sup> (R, A or G; Y, T or C; M, C or A; N, A or C or G or T)

<sup>b</sup> (cc, clamp; gaattc, EcoRI restriction site; ggatcc, BamHI restriction site)

Table II  
Expression of HexA C5-epimerase in Sf9 cells

Sf9 cells ( $1 \times 10^6$  in 4 ml medium) were seeded in 60-mm Petri dishes and incubated for three hours at 27°C. After the cells were attached, the medium was removed, and 200  $\mu$ l of recombinant virus stock was added to infect the cells at room temperature for 1h. The virus suspension was aspirated and 4 ml of medium was added to each dish. The cells were incubated at 27 °C for 5 days. The medium was transferred into a steril tube and centrifuged. The cells were collected, washed twice with PBS and lysed with 300  $\mu$ l of homogenization buffer as described under "Experimental Procedures". Aliquots (25  $\mu$ l) of cell lysate and medium were assayed for epimerase activity. The activity is expressed as release of  $^3\text{H}$  from K5 polysaccharide per hour. The data is mean value of three independent assays.

Construct	Epimerase Activity	
	Cell lysate (cpm/mg/h)	Medium (cpm/ml/h)
HexA C5-Epimerase-1	102670 $\pm$ 5540	45200 $\pm$ 1770
HexA C5-Epimerase-2	123270 $\pm$ 4660	52610 $\pm$ 810
HexA C5-Epimerase-1 (heat-inactivted)	240	610
N-Deacetylase/sulfotransferase	9520 $\pm$ 620	1350 $\pm$ 280
$\beta$ -Glucuronidase	8460 $\pm$ 1270	1610 $\pm$ 440
BacPAK plasmid	5150 $\pm$ 880	2820 $\pm$ 690
Neo	7250 $\pm$ 370	550 $\pm$ 120

[illegible][illegible]

CTCGGGAAAGAGCGAGGTCCTTGTATGAGCGTGCCATGGAAATCCCTTAAAGCCATGCTC 1200  
 L G K E A R S L Y E R G M E S L K A M L 376  
 CCCTTGTACGACACTGGCTCAGGAACCATCTATGACCTCCGGCAGTTTCATGCTTGGCATT 1260  
 P L Y D T G S G T I Y D L R H F M L G I 396  
 GCGCCCAACTGGCCCCGCTGGGACTATCACACCCACATCAATCAACTGCAGCTGCTT 1320  
 A P N L A R W D Y H T T H I N Q L Q L L 416  
 AGCACCATTTGATGAGTCCCAATCTTCAAGAATTTGTCAAGAGGTGGAGAAGCTACCTT 1380  
 S T I D E S P I F K E F V K R W K S Y L 436  
 AAGGCGAGCGCGCAAGCACAACCTAGAGCTCAGAACCAAAATCCTACGTCAGCCTCTGC 1440  
 K G S R A K H N 444  
 TGTACACAGAACTAGAGGCTCTGTGTCAACAGGCATAGGCACATTTTAAAGGCTGTA 1500  
 TACTAGGTTTTTGTGGATTACATCAAAGTGATAAATGATCCTTAAAGCCAGTCTTCTGAG 1560  
 ATAATTGCATTCATCGGTTTGTAGTGTTCGAATGTCGATGCCATTTATAGCAGAAAGTG 1620  
 TTTAGTCAGTGGGCTGAATGAAGATGTTTAACTTGGGCTCGCTTATCAGCCTGTTCAGTT 1680  
 CCACAGGTAGTCCAGTTCTCTCGATTGTGGAAAGACAATGGTAAGTAGCTCTTCATGGCC 1740  
 AGCTGTCCAGCACTTGTCTGAAAACCTAGTATGGGGCTCTTTTAAAGTGTGGTTATTTAT 1800  
 GTTTATGTTGAAAGCAGACTTTAAAAAATPATGTGCTAAAATACAGTAAATATGTACTT 1860  
 GTAGCCTGATAGTGACTGTGTGCAACTTTAAAAATGATTTTCTTTTCTATAAATTAATT 1920  
 TCTTAGCGGTGGATGAGCAATTTGTGTGTTTGTTCAGTTGTTATATATGGAGAATATTT 1980  
 TGAATTTATGGTTTGTCTGAAGTGTATAAATTA AAAACACAACCAAGTGTTCAGGCTTCAC 2040  
 AGTTATATAATGTAAGCACAACCTAAAATGAACTTGTGTGACTGCACAAGAAATTACAAAA 2100  
 CAGAACAAAATGTTATCTGTTTATGAACTATCTACAATCAGTAAAGATTTGATAATC 2160  
 AGTATACCCCTCCGTACCCCATTTGTGGTGGTTTCTTTTGGCCACTATCTCAAAATTTG 2220  
 TATTTCAATTCAGACTACACTTGAGAGTTTTTGTCTATTTTGGGGGGACATTTTGGGGACA 2280  
 TTTGGGAATTTTACTATAAACCTAGATTTGATGAGGAGGTAGTAAGTTTAATAAGCCCA 2340  
 CTACCACTGGCTTTTCTAGATTCTTTTCCCTTTAAGGAAAAATATTAGGTACAGATATTA 2400  
 TAAGGATTGTAGCAGATTTTTCCTACTTAGATCATTCTTGGTCTACAGCTTTCCAAC 2460  
 TATTGATGTACACAAAATACATAGTTTTTGTGTAGCTTTCAAACCTTTCTGGTGTTTTT 2520  
 TCTTTGAGTTTTTAAATTTTAAATTTATTTACCTCTTGGATAAAAGTGATCTACTATAT 2580  
 TAGCTGTACATGTGTAAATCAGACCTTTATTTTGGTTTTATATCCACATACTCACAATA 2640  
 ATAGGCATCATAGCCCTCACACCCCTGGCCAGTGTCTGCTCTAGGACTTAGGCAGTAGGTC 2700  
 AGAAGTGAAGGAGGTTGATTTTGTCTGTCTGTTTTAGTGTATGACAATACAGTAAATCA 2760  
 ATACAATAACTTATACAGATTGGAAATACGAGATCCGTAATTTTCAGAGGACTGAGTCTG 2820  
 ACAACCCAGTCCAGTGTGTGTGTGACCTGTATGAAATGCACATCAAGAGCGAGGTGGCA 2880  
 CCTGCTGCCACTGCATCTTGGCTGGACTTAGTCTACCAACACCACTCAGAAATGGCAAA 2940  
 ATGCATACATGCTTTTGAOACAATATATGTTGTATCAGCAGCCGGAACCAAGACCTACA 3000  
 ACTGACATGAAGTGTAGTCACTAAGTGTGTCCAACCTCTTTGTGACCTCATAGACTGT 3060  
 AGCCCGCCAGGCTTCTTTGTCCATG 3085

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ART 34

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CLAIMS

5           1. An isolated or recombinant DNA sequence coding  
for a mammalian, including human, glucuronyl C5-epimerase,  
or a functional derivative of said DNA sequence, capable of  
converting D-glucuronic acid (GlcA) to L-iduronic acid  
(IdoA) constituted by a nucleotide sequence comprising nu-  
10 cleotide residues 1 to 1404, inclusive, as depicted in the  
sequence listing.

2. A DNA sequence according to claim 1 consti-  
tuted by a nucleotide residue comprising nucleotide resi-  
dues 73 to 1404, inclusive, as depicted in the sequence  
15 listing.

3. A DNA sequence according to claim 2 consti-  
tuted by a nucleotide residue comprising nucleotide resi-  
dues 1 to 1404, inclusive, as depicted in the sequence  
listing.

20           4. A recombinant expression vector containing a  
transcription unit comprising a DNA sequence according to  
any one of the preceding claims, a transcriptional pro-  
moter, and a polyadenylation sequence.

5. A recombinant expression vector according to  
25 claim 4, characterized in that the vector is a Baculovirus.

6. A host cell transformed with the recombinant  
expression vector of claim 4 or 5.

7. A process for the manufacture of a glucuronyl  
C5-epimerase or a functional derivative thereof capable of  
30 converting D-glucuronic acid (GlcA) to L-iduronic acid  
(IdoA), comprising cultivation of a host cell transformed  
with a recombinant expression vector according to claim 4  
or 5 in a nutrient medium allowing expression and secretion

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25-05-1999

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of said epimerase or functional derivative thereof.

- 5 8. A glucuronyl C5-epimerase (or a functional de-  
rivative) thereof whenever prepared by the process of claim  
7.

SECRET

AMENDED SHEET

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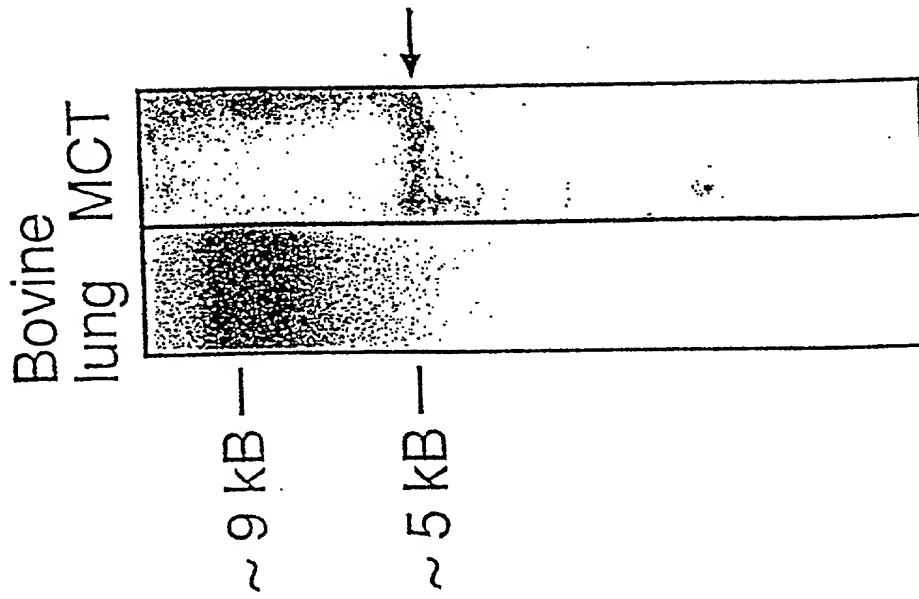


Figure 3

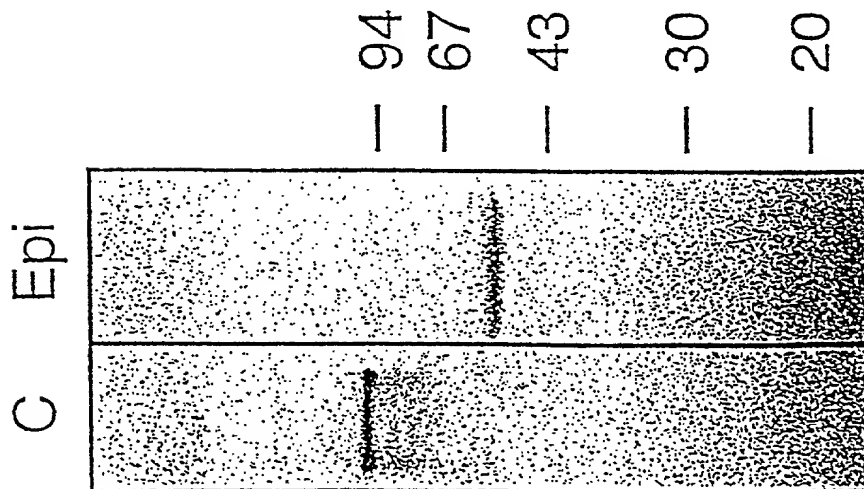


Figure 1

Publ. No. 9820460

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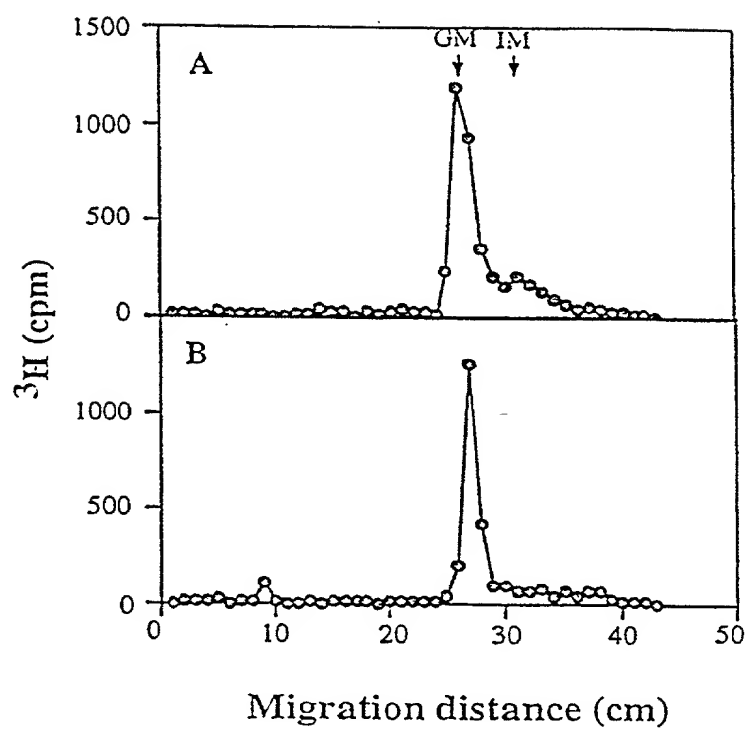


Figure 2

**COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR UTILITY PATENT APPLICATION**

Attorney's Docket No.

003300-589

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (if only one name is listed below) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (if more than one name is listed below) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED:

NEW DNA SEQUENCES CODING FOR A MAMMALIAN GLUCORONYL C5-EPIMERASE  
AND A PROCESS FOR ITS PRODUCTION

the specification of which

(check one)

☐ is attached hereto;

☒ was filed on 17 April 1998 as

Application No. PCT/SE98/00703

and was amended on 25 May 1999;  
(if applicable)

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE;

I ACKNOWLEDGE THE DUTY TO DISCLOSE TO THE OFFICE ALL INFORMATION KNOWN TO ME TO BE MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS, Sec. 1.56 (as amended effective March 16, 1992);

I do not know and do not believe the said invention was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said application; that said invention was not in public use or on sale in the United States of America more than one year prior to said application; that said invention has not been patented or made the subject of an inventor's certificate issued before the date of said application in any country foreign to the United States of America on any application filed by me or my legal representatives or assigns more than twelve months prior to said application;

I hereby claim foreign priority benefits under Title 35, United States Code Sec. 119 and/or Sec. 365 of any foreign application(s) for patent or inventor's certificate as indicated below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application(s) on which priority is claimed:

## COMBINED DECLARATION AND POWER OF ATTORNEY

Attorney's Docket No.

COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
Sweden	9701454-2	18 April 1997	YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

William L. Mathis	17,337	James A. LaBarre	28,632	Marthaw L. Schneider	32,814
Peter H. Smolka	15,913	E. Joseph Gess	28,510	Michael G. Savage	32,596
Robert S. Swecker	19,885	R. Danny Huntington	27,903	Gerald F. Swirs	30,113
Platon N. Mandros	22,124	Eric H. Weisblatt	30,505	Michael J. Ure	33,089
Benton S. Duffett, Jr.	22,030	James W. Peterson	26,057	Charles F. Wieland III	33,086
Norman H. Stepno	22,716	Teresa Stanek Rea	30,427	Bruce T. Wieder	33,815
Ronald L. Grudziecki	24,970	Robert E. Krebs	25,883	Todd R. Walters	34,040
Frederick G. Michaud, Jr.	25,003	William C. Rowland	30,888	Ronni S. Jillions	31,979
Alan E. Kopecki	25,813	T. Gene Dillahunty	25,423	Harold R. Brown III	36,341
Regis E. Slutter	26,999	Patrick C. Keane	32,858	Allen R. Baum	36,086
Samuel C. Miller III	27,360	Bruce J. Boggs, Jr.	32,344	Steven M. du Bois	35,023
Ralph L. Freeland, Jr.	16,110	William H. Benz	25,952	Brian P. O'Shaughnessy	32,747
Robert G. Mukai	28,531	Peter K. Skiff	31,917		
George A. Hovanec, Jr.	28,223	Richard J. McGrath	29,195		

and:

Address all correspondence to:

Benton S. Duffett, Jr.  
 BURNS, DOANE, SWECKER & MATHIS, L.L.P.  
 P.O. Box 1404  
 Alexandria, Virginia 22313-1404



21839

PATENT TRADEMARK OFFICE

Address all telephone calls to: Benton S. Duffett, Jr. at (703) 836-6620.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF SOLE OR FIRST INVENTOR Ulf Lindahl		SIGNATURE <i>Ulf Lindahl</i>	DATE Oct. 9, 1999
RESIDENCE UPPSALA, Sweden		CITIZENSHIP Swedish	
POST OFFICE ADDRESS Torgvägen 7, S-756 46 UPPSALA, Sweden			
FULL NAME OF SECOND JOINT INVENTOR, IF ANY Jin-ping Li		SIGNATURE <i>Jin-ping Li</i>	DATE Oct. 9, 1999
RESIDENCE UPPSALA, Sweden		CITIZENSHIP Swedish	
POST OFFICE ADDRESS Reykjaviksgatan 51, S-752 63 UPPSALA, Sweden			
FULL NAME OF THIRD JOINT INVENTOR, IF ANY		SIGNATURE	DATE
RESIDENCE		CITIZENSHIP	
POST OFFICE ADDRESS			